



Exhibit 3

Ion conduction pore is conserved among potassium channels

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Potassium channels, a group of specialized membrane proteins, enable K⁺ ions to flow selectively across cell membranes. Transmembrane K⁺ currents underlie electrical signalling in neurons and other excitable cells. The atomic structure of a bacterial K⁺ channel pore has been solved by means of X-ray crystallography. To the extent that the prokaryotic pore is representative of other K⁺ channels, this landmark achievement has profound implications for our general understanding of K⁺ channels. But serious doubts have been raised concerning whether the prokaryotic K⁺ channel pore does actually represent those of eukaryotes. Here we have addressed this fundamental issue by substituting the prokaryotic pore into eukaryotic voltage-gated and inward-rectifier K⁺ channels. The resulting chimaeras retain the respective functional hallmarks of the eukaryotic channels, which indicates that the ion conduction pore is indeed conserved among K⁺ channels.

Potassium channels are a highly diversified group of tetrameric integral membrane proteins. Voltage-gated K⁺ channels (K_v) underlie electrical impulse generation in nerve, muscle and endocrine cells, whereas inward-rectifier K⁺ (Kir) channels help to maintain and regulate resting membrane potential. K_v channel subunits have six transmembrane segments, whereas Kir subunits have two^{1–5}. The atomic structure of a bacterial K⁺ (KcsA) channel pore has been solved by X-ray crystallography⁶. To the extent that the pore of KcsA is representative of other classes of K⁺ channels, the implication of this achievement for a general understanding of K⁺ channels is obvious.

Although KcsA and Kir subunits share a two-transmembrane-helix topology, their primary structures are barely conserved^{4,5,7}, and serious doubts about the generality of the KcsA pore among K⁺ channels have been raised. For example, it has been suggested, on the basis of a genetic study, that the arrangement of transmembrane segments of eukaryotic Kir and of prokaryotic KcsA is distinctly different⁸. It has also been proposed that a sharp bend—presumably occurring at a conserved Pro-x-Pro sequence in the sixth transmembrane segment (S6)—makes the inner pore of K_v channels different from that of KcsA, with significant implications for channel gating⁹.

Substitution of the KcsA pore in a K_v channel

To assess the generality of the KcsA pore, we produced a chimaeric channel, KcsA–Shaker, in which the prokaryotic KcsA pore replaces that of the eukaryotic Shaker K_v channel with its inactivation particle removed¹⁰ (Fig. 1a). The current through the chimaeric channels is both K⁺ selective and voltage gated (Fig. 1b–e; 10 mM K⁺ in extracellular solution). Although the chimaera's gating parameters differ quantitatively from those of Shaker itself (the depolarization-induced current develops more slowly than in Shaker; $V_{1/2}$ of the activation curve is about +10 mV compared with less than –30 mV for Shaker; and its apparent valence is 1.5 compared with more than 3 for Shaker)^{11,12}, they resemble those of Shaker channels that contain certain point mutations. For example, $V_{1/2}$ and the apparent valence of the activation curve for Shaker channels containing a leucine to tryptophan mutation at residue 398 are about +20 mV and 1.8, respectively¹¹.

Extracellular agitoxin 2 (AgTx2) blocks Shaker, but not KcsA channels^{13,14} or our KcsA–Shaker construct (Fig. 2c). But by replacing Gln 58 of KcsA with alanine, and Thr 61 and Arg 64 with their counterparts in Shaker's P-loop (Ser 428 and Asp 431), KcsA is rendered sensitive to AgTx2 (ref. 14). Thus, if the attached Shaker

sequences do not significantly alter the pore structure of the chimaera, then a KcsA–Shaker construct with the three point mutations (KcsA_{3M}–Shaker) should also be sensitive to AgTx2. This is verified in Fig. 2b, d and e, which shows that KcsA_{3M}–Shaker is 20 times more sensitive than Shaker to AgTx2.

The inward current at the –100 mV holding potential in oocytes expressing KcsA–Shaker channels, which is minimal in 10 mM extracellular K⁺ (Figs 1b and 3b) but prominent in 100 mM K⁺ (Fig. 3d), is larger than the expected background leak current in oocytes not injected with RNA. This observation, and the fact that the inward current at –100 mV in KcsA_{3M}–Shaker chimaeras is sensitive to AgTx2 (Fig. 3f) argue that the chimaeric channels still conduct at very negative potentials—a feature resembling that of a P475D Shaker mutant whose activation gate is suggested to be disrupted¹⁵. This raises the question of whether our chimaeric construct is only partially voltage gated (that is, cannot be fully closed) or whether hyperpolarization beyond experimental reach is required to close it completely. Although this uncertainty remains, KcsA–Shaker with an alanine mutation at the position corresponding to Gly 104 in KcsA opens at more depolarized potentials and carries no significant current at –100 mV with either low or high extracellular K⁺ concentration (Fig. 3c, e, g). This finding confirms that the chimaeric construct is intrinsically capable of being fully gated by membrane voltage. In addition, the current carried by KcsA–Shaker decreases (less so with the G104A mutation) as extracellular K⁺ is lowered, a dependence observed previously with other K_v channels¹⁶, which may result from channel block and/or a conformational change as in the case of C-type inactivation^{17–19}.

Substitution of the KcsA pore in a Kir channel

Whereas the amino-acid sequence that forms the pore is well conserved between K_v and KcsA, it is markedly different in Kir, as illustrated for the amino-terminal portion of the P-loop, which is defined here as the entire linker between the first and second transmembrane segments (M1 and M2) in Kir and KcsA, or its counterpart between S5 and S6 in K_v channels (Fig. 4a). Alignment shows that the P-loops in Kir differ from those in K_v and KcsA not only in residue composition, but also in length on account of an extra, variable segment that presumably forms the turrets lining the external vestibule in Kir⁶. However, deletion of this 'insert' from IRK1 (Kir 2.1), which reduces the length of its P-loop to that of KcsA and K_v, has little effect on either voltage-jump-induced relaxation or the degree of rectification (Fig. 4b, c).

More direct evidence for pore conservation between KcsA and Kir

comes from a chimaera, KcsA-IRK1, in which we replaced the pore of IRK1 with that of KcsA (Fig. 5a, b). Like KcsA²⁰, the chimaera is blocked by extracellular tetraethylammonium (TEA) (dissociation constant, $K_d = 0.8 \text{ mM}$) with little voltage dependence (Fig. 5d, f, h, i). The fact that KcsA-IRK1 rectifies only modestly compared with IRK1 is probably due to the absence in KcsA of an aspartate residue that is present in the M2 region of IRK1 and is crucial for the voltage-dependent high-affinity binding of intracellular blocking cations that underlies strong inward rectification²¹⁻²⁵.

Because replacing a neutral residue with aspartate in the M2 region of weakly rectifying ROMK1 (Kir 1.1) is known to enhance markedly that channel's affinity for intracellular cations and hence rectification^{26,27}, we made such a substitution in KcsA-IRK1. The M2 residue replaced, which corresponds to Thr 107 in KcsA, sits at the intracellular end of the cavity that separates the outer K^+ -selective part from the inner part of the pore, its side chain pointing into the cavity⁶. The T107D mutation does convert KcsA-IRK1 into a strong inward rectifier (Fig. 5c). The small outward current in the

oocyte expressing T107D channels is not reduced much by extracellular TEA at concentrations that strongly block the much larger inward current, and must therefore be background current (Fig. 5e, g). This signifies that T107D channels themselves carry insignificant outward current. Current relaxation in T107D channels after membrane hyperpolarization is much slower than in IRK1—a feature seen in some other strong rectifiers including GIRK1/4 (Kir 3.1/Kir 3.4)²⁸⁻³⁰ and BIR10 (Kir 4.1)³¹.

Mutation T107D in the KcsA pore not only enhances rectification in KcsA-IRK1 but also renders voltage-gated KcsA-Shaker inwardly rectifying. At a holding potential of 0 mV, KcsA_{3M}-Shaker exhibits significant (AgTx2-sensitive) conductance, which undergoes further activation with time at positive voltages and considerable deactivation at negative voltages (Fig. 6b, d). In contrast, KcsA_{3M}-Shaker containing T107D carries large AgTx2-sensitive inward currents but minimal outward currents; no deactivation is observed at negative voltages (Fig. 6c). This point mutation therefore converts a delayed rectifier into an inward rectifier (Fig. 6b, d, compare c, e), although the extent of intrinsic voltage gating of the T107D-containing channels, if any, remains to be determined. Thr 107 corresponds to Val 474 of Shaker in the conserved Pro-x-Pro sequence of classic K_v channels⁹ (Fig. 6a). Evidently, when examined under comparable conditions, KcsA pores with the T107D mutation conduct K^+ in a comparable manner whether they are inserted into an IRK1 or a Shaker sequence (compare Figs 5 and 6).

Discussion

Our findings show that the pore of KcsA can substitute for that of K_v or Kir. Therefore, although they differ fundamentally in both overall architecture (two compared with six transmembrane segments) and gating properties (K_v is gated by voltage, KcsA by protons^{20,32}, and Kir by PIP₂ (ref. 33) and other messenger molecules), these K^+ channels must share a conserved pore. Our argument for a conserved pore is consistent with the previously reported promis-

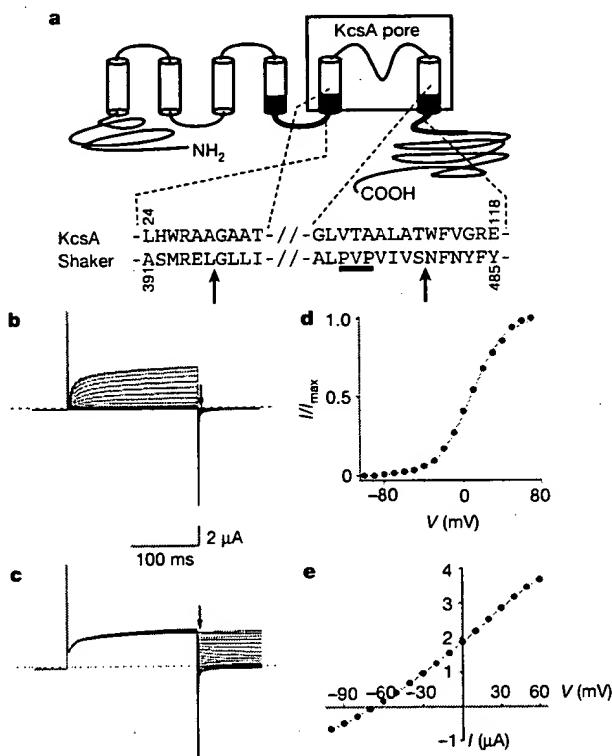


Figure 1 Replacement of the Shaker pore module with that of KcsA. **a**, Representation of the polypeptide chain topology of the KcsA-Shaker chimaeric channel and partial sequences of the parent channels around the splicing sites. In the chimaera, the S5-S6 region of Shaker (indicated between the arrows) is replaced by the corresponding part from KcsA. Black regions, putative interaction regions (see Discussion). **b, c**, KcsA-Shaker currents recorded in 10 mM K^+ solution from the same oocyte, as the membrane potential was stepped from the -100 mV holding potential either to test potentials 10 mV apart between -100 and +70 mV, and back (**b**), or to +60 mV and then test potentials between -100 and +60 mV (**c**). Dashed line identifies the zero current level. **d, e**, Currents in **b** (normalized) and **c**, respectively, at the time indicated by arrows are plotted against voltage. As an approximation, data in **d**, where current at -100 mV is set as zero and that at +70 mV as I_{max} , are fitted with the equation

$$\frac{I}{I_{max}} = \frac{1}{1 + \exp\left(-\frac{zF(V - V_{1/2})}{RT}\right)}$$

(where F , R and T are Faraday's constant, the gas constant and absolute temperature, respectively) yielding valence $z = 1.5$ and midpoint voltage $V_{1/2} = +7 \text{ mV}$.

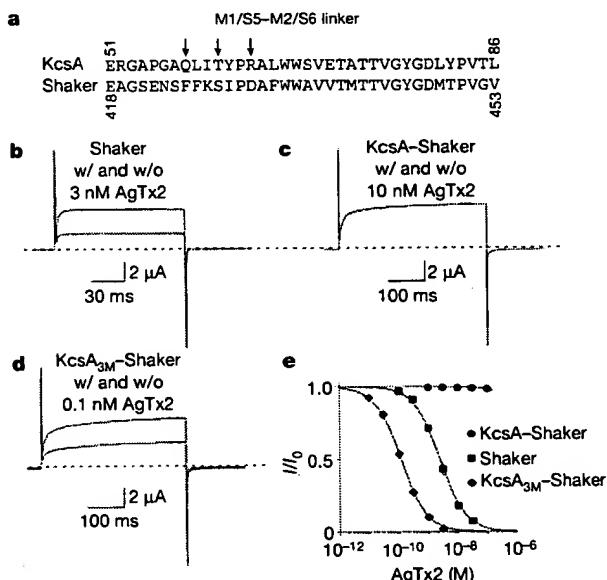


Figure 2 Triple mutation in the P-loop renders KcsA-Shaker channels sensitive to AgTx2. **a**, Alignment of the KcsA and Shaker P-loops. Arrows indicate the sites of the three point mutations described in the text. **b-d**, Currents through Shaker, KcsA-Shaker or KcsA_{3M}-Shaker channels with and without AgTx2, recorded as the membrane potential was stepped from -80 mV to +20 mV and back. **e**, Fractions of unblocked currents (mean \pm s.e.m., $n = 6-7$) versus AgTx2 concentration, where I_0 is the current in the absence of AgTx2. The curves through the data of Shaker and KcsA_{3M}-Shaker are fits of the equation $\frac{I}{I_0} = K_d / (K_d + [\text{AgTx2}])$, with $K_d = 2.38 \pm 0.12 \text{ nM}$ (mean \pm s.e.m., $n = 6$) and $0.11 \pm 0.01 \text{ nM}$ ($n = 7$), respectively.

ciuity displayed by the external pore-blocking scorpion toxins among various classes of K^+ channels, including K_v , Kir and KcsA^{14,24}. Some differences among K^+ channel pores must undoubtedly remain. For example, the pore of IRK1 may be extended intracellularly by part of the carboxy terminus beyond the essential, conserved pore-forming module^{25,35}. In addition, a conserved

Pro-x-Pro sequence in the S6 region of classic K_v channels may confer specific features to those channels⁹, although it is not essential for voltage gating because KcsA-Shaker, which lacks this sequence, is still voltage gated (Fig. 3).

Our chimaeric channel constructs illustrate experimentally the modular nature of K_v channel architecture, namely, a pore-forming module plus one or more voltage-sensing modules^{4,5,7,11,36}. This modular architecture implies that voltage-gated K^+ channels evolved from KcsA or similar channels and that a separate KcsA (voltage or non-voltage) gating module, not attached to the pore

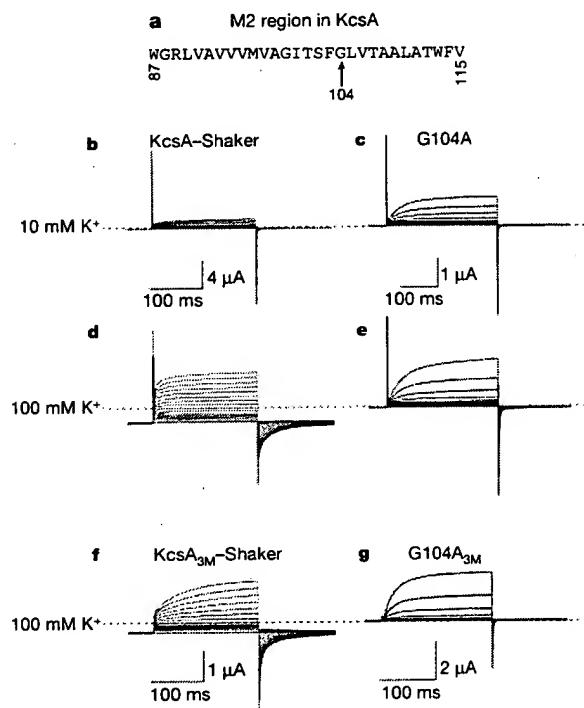


Figure 3 Effects of extracellular K^+ concentration and a G104A mutation on the gating of KcsA-Shaker. **a**, Amino-acid sequence of the M2 region of KcsA. **b–e**, Currents through KcsA-Shaker without and with an alanine mutation at the residue corresponding to G104 of KcsA in the absence or presence of two concentrations of extracellular K^+ . Records shown in **b** and **d**, or in **c** and **e** were recorded from the same oocyte. **f, g**, Currents recorded through KcsA_{3M}-Shaker channels without and with the G104A mutation are corrected for background current with AgTx2 as described in Methods. The voltage protocol was the same as used in Fig. 1b.

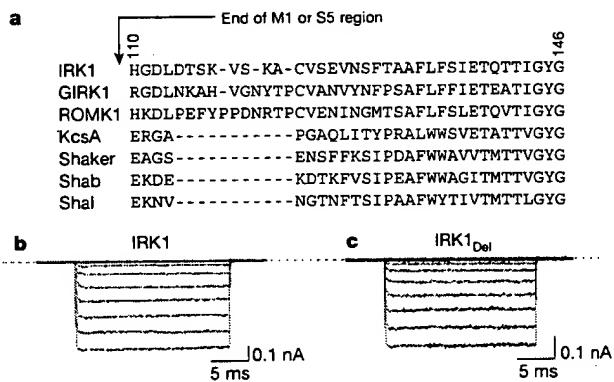


Figure 4 Deletion of a segment in the P-loop of the IRK1 channel. **a**, Alignment of the partial P-loop from N-terminal end⁴ to Gly-Tyr-Gly among various K^+ channels^{2–5,7,28,29,50}. **b, c**, Currents of IRK1 and of its mutant lacking the sequence between His-Gly-Asp-Leu and Cys-Val-Ser-Glu (IRK1_{Del}), corrected for background leak currents as described in Methods. To resolve the relatively rapid current relaxation kinetics in these channels, we recorded from cell-attached membrane patches rather than from whole oocytes (which were used in the other figures). During the recording, membrane potential was stepped from the 0 mV holding potential to test potentials 10 mV apart between –80 and +80 mV, and then back to 0 mV.

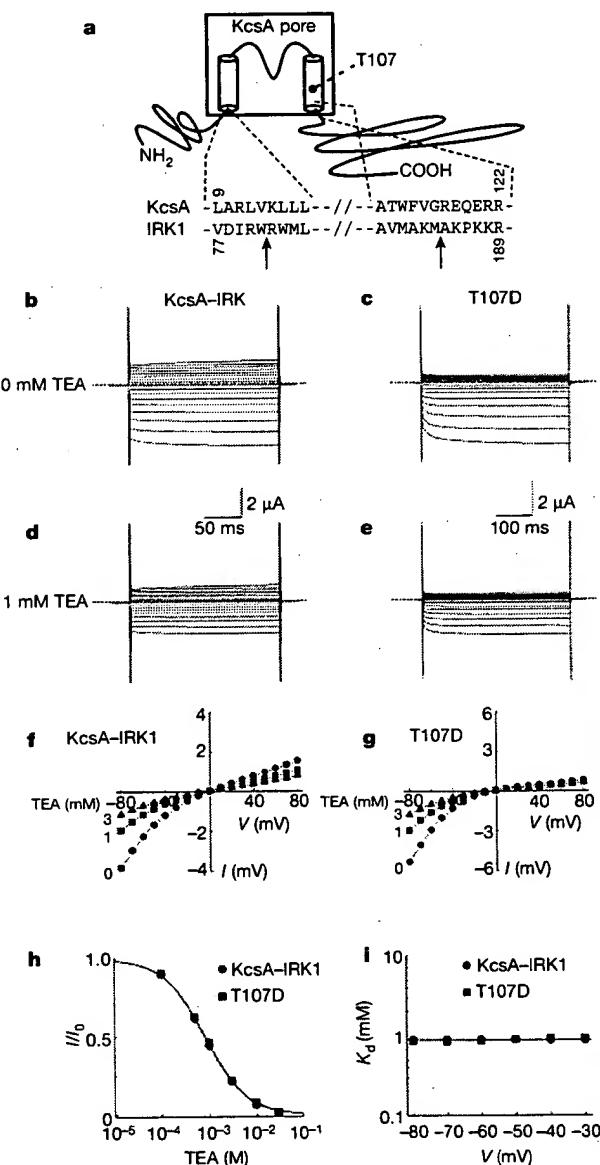


Figure 5 Substitution of the KcsA pore in IRK1. **a**, Peptide chain topology of the KcsA-IRK1 chimaera channel and partial sequences of the parent channels around the splicing sites. In the chimaera, the M1-M2 region of IRK1 (indicated between the arrows) is replaced by the corresponding part from KcsA. **b–g**, Currents through KcsA-IRK1 (**b**, **d**) and its T107D mutant (**c**, **e**), without (**b**, **c**) or with (**d**, **e**) extracellular TEA, were recorded using the voltage protocol of Fig. 4; the corresponding I - V curves are plotted in **f** and **g**. **h**, Fractions of unblocked currents (mean \pm s.e.m., $n = 5$) at –80 mV are plotted against TEA concentration; the curves are fits of the equation $I/I_0 = K_d / (K_d + [TEA])$ where $K_d = 0.81 \pm 0.02$ mM for KcsA-IRK1 and 0.86 ± 0.02 mM for T107D. **i**, K_d (mean \pm s.e.m., $n = 5$) determined from fits similar to (and including) those shown for –80 mV in **h** are plotted against membrane voltage.

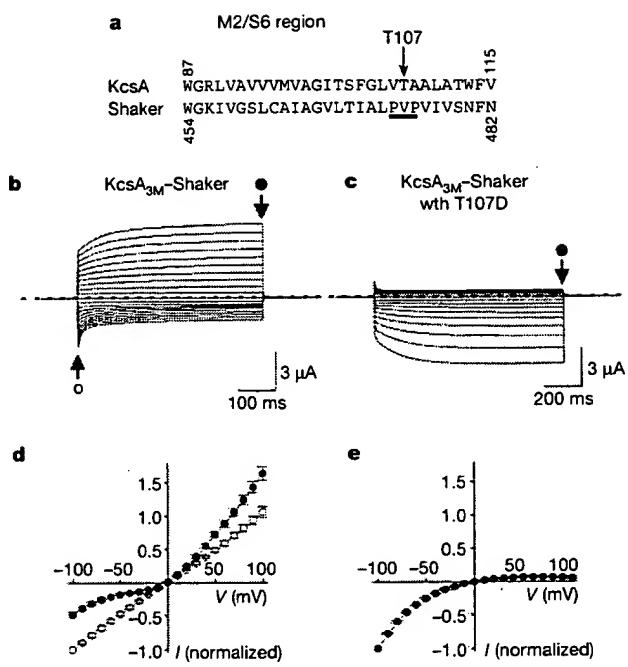


Figure 6 Mutation T107D renders KcsA-Shaker inwardly rectifying. **a**, Alignment of M2/S6 sequences of KcsA and Shaker. **b**, **c**, Current records of KcsA_{3M}-Shaker without and with T107D in the presence of 100 mM extracellular K⁺. During the recording, membrane potential was stepped from the 0 mV holding potential to test potentials 10 mV apart between -100 and +100 mV, and then back to 0 mV. Correction for background currents was done with AgTx2 as described in Methods. **d**, **e**, I-V curves constructed from data similar to (and including) those shown in **b** and **c**, at the time indicated by arrows and with the corresponding symbols. Each data point represents mean current (\pm s.e.m., $n = 13$ (**d**) and $n = 12$ (**e**)) normalized to that at -100 mV. The AgTx2-insensitive outward current in oocytes injected with cDNA of KcsA_{3M}-Shaker containing T107D is significantly larger than in uninjected oocytes, and unaffected by replacing 100 mM extracellular K⁺ with 100 mM Na⁺. This current may reflect the oocyte's response to expression of the channels.

module through the main peptide chain, may exist in *Streptomyces lividans*. Furthermore, our demonstration that attaching the voltage-sensing module of Shaker to the pore of KcsA confers voltage gating on the latter has important implications regarding the mechanics of voltage gating.

For example, the voltage-sensing module has been suggested to couple the voltage sensor to the channel gate through a specific set of residues in the shell of the pore-forming module of K_v (ref. 11). If so, the voltage sensitivity of our KcsA-Shaker chimaera would argue that the shell of KcsA's pore (which is presumed normally to form an interface with membrane lipids) must be constructed in such a way that it, too, can establish a functional interface that mediates coupling between the voltage-sensing module(s) and the channel gate. Considering that our voltage-gated KcsA-Shaker chimaera contains KcsA's pore module (S5-S6) but Shaker's S4, S4-S5 linker and C terminus, primary coupling between the voltage-sensing S4 (refs 37-42) and the internal channel gate probably occurs more intracellularly. In this coupling scheme, residues in the sequence encompassing the C-terminal part of S4 to the N-terminal part of S5 interact with specific residues in the C-terminal part of S6 and its extension where the intracellular gate is located^{43,44} (the two putative interacting regions are highlighted in grey).

Consistent with this view, a study has shown that a KcsA-Shaker that differs from ours mainly in that it contains KcsA's C terminus, whereas ours has Shaker's, does not conduct any current over a range of voltage and pH even though it reaches the

membrane⁴⁵. Furthermore, most of the distal portion of the C terminus in a K_v channel is non-essential for voltage gating⁴⁶. If the C-terminal part of S6 and its extension (or their counterparts in other K⁺ channels) indeed act as the 'door handle', then this would explain why Kir, KcsA and Ca²⁺-activated K⁺ channels are gated by intracellular molecules, whereas K⁺ channels with inside-out membrane topology⁴⁷, such as GluR0, are gated extracellularly.

Methods

Molecular biology and channel expression

Complementary DNAs for Shaker H4 with N-type inactivation removed^{4,10} and IRK1 (ref. 5) were cloned in pBluescript and/or pGEM-HESS plasmids, respectively. To replace the DNA sequence encoding the pore of Shaker or IRK1 with that of KcsA, we subcloned a polymerase chain reaction (PCR)-generated DNA fragment containing the KcsA segment of interest into the recipient version of Shaker or IRK1 cDNA. The N- and C-terminal junctions in the KcsA-Shaker construct are respectively formed by joining Leu 396 of Shaker to Gly 30 of KcsA (ref. 7; residue 397 of Shaker is also glycine) and Thr 112 of KcsA to Asn 480 of Shaker; those in the KcsA-IRK1 construct are respectively formed by joining Trp 81 of IRK1 to Lys 14 of KcsA and Gly 116 of KcsA to Ala 184 of IRK1. The cDNAs encoding the remaining mutant channels were also produced using PCR mutagenesis. We confirmed all PCR-generated sequences by DNA sequencing. All cRNA were synthesized with T7 polymerase using linearized cDNA as templates. Oocytes were prepared and injected with cRNAs as described⁴⁸.

Recordings of channel currents

All channel currents were recorded from whole oocytes using a two-electrode voltage-clamp amplifier (Warner OC-725C), except those shown in Fig. 4, which were recorded from cell-attached membrane patches using a patch-clamp amplifier (Axopatch 200B) to resolve the relatively rapid current relaxation kinetics of the channels. The resistance of electrodes filled with 3 M KCl was 0.4 MΩ. Unless specified otherwise, the bath solution for studying Shaker and KcsA-Shaker contained (in mM): 90 Na⁺ (Cl⁻ + OH⁻), 10 KCl, 0.3 CaCl₂, 1 MgCl₂ and 10 HEPES; pH 7.6 with NaOH. When the K⁺ concentration was increased, the Na⁺ concentration was decreased so that their sum remained 100 mM. In the case of KcsA_{3M}-Shaker (with and without G104A or T107D) the background currents were obtained in the presence of AgTx2 at concentrations greater than 100 nM. The concentration of AgTx2 stock was calculated using a published extinction coefficient (7.48 mM⁻¹ cm⁻¹ at 235 nm)¹⁹. The solution for studying IRK1 and IRK1-KcsA contained (in mM): 100 K⁺ (Cl⁻ + OH⁻), 0.3 CaCl₂, 1 MgCl₂ and 10 HEPES; pH 7.6 with KOH. We recorded the currents shown in Fig. 4 with a patch-clamp amplifier in the cell-attached configuration. After recording, the membrane patch was excised and its inside was exposed to a solution containing 3 mM Mg²⁺ to induce a rapid channel rundown²⁰. The currents recorded after channel rundown were used as templates for the subsequent off-line background current correction. The pipette and bath solutions contained (in mM): 5 K₂EDTA, 10 K₂HPO₄ + KH₂PO₄ in a ratio yielding pH 7.6, and sufficient KCl to bring total K⁺ concentration to 100 mM (ref. 49).

Received 1 June; accepted 29 August 2001.

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Acknowledgements

We thank P. De Weer for review and discussion of our manuscript; C. M. Armstrong, R. MacKinnon and K. J. Swartz for discussions and suggestions; R. MacKinnon and E. Perozo for KcsA cDNA; K. J. Swartz for Shaker-IR cDNA subcloned in the pGEM-HESS vector; L. Y. Jan for IRK1 cDNA; and J. Yang for IRK1 cDNA subcloned in the pGEM-HESS vector. This study was supported by the National Institute of General Medical Sciences. Z.L. is the recipient of an Independent Scientist Award from the National Heart, Lung and Blood Institute.

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